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14. ABSTRACT <i>Enteromorpha</i> is the most important alga fouling hulls, whether coated with conventional Cu-based paints or foul-release silicones. Since fouling by <i>Enteromorpha</i> is increasing as a consequence of the increased deployment of TBT-free coatings, it is important to understand the processes involved in the initiation of fouling, i.e. spore settlement and adhesion. Our results provide the most comprehensive characterisation of the settlement and adhesion processes and the roles of surface-associated cues, of any soft-fouling species to date. We have shown that spores respond to chemical, physico-chemical, biological (microbial biofilm) and topographic cues. Novel insights have been gained on communication processes involved in the development of a micro-fouling community. Thirteen papers in refereed journals and several abstracts were published.					
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FINAL REPORT

Grant #: N00014-96-0373

PRINCIPAL INVESTIGATORS: Prof. J A Callow and Dr Maureen E Callow

INSTITUTION: University of Birmingham, UK

GRANT TITLE: 1) Primary adhesion in *Enteromorpha*. 2) Cue detection and surface selection in the settlement and adhesion of *Enteromorpha* spores.

AWARD PERIOD: 1 December 1995 - 30 November 2001

OBJECTIVES: Our long-term goal is to prevent fouling of marine underwater structures, specifically ships and submarines, by the green alga *Enteromorpha*. Towards this goal this project aimed to 1) characterize settlement and adhesive processes used by *Enteromorpha*; 2) develop adhesion microassays and use these to explore adhesion phenomena quantitatively; 3) examine the detailed behaviour of zoospores during settlement and attachment to various substrata using video microscopy; 4) identify and evaluate the role of diffusible chemical and topographical settlement cues; 5) evaluate the importance of the microbial biofilm as a source of such signals; 6) evaluate the impact of different surface chemistries and physical properties on spore settlement and attachment; 7) extend the analyses of the influence of these chemical, physical and biological factors on primary adhesion, to their impact on subsequent development and establishment of sporelings.

APPROACH: Quantitative assays of zoospore settlement and adhesion were developed and used to analyse the kinetics of adhesion to test surfaces and to test the influence of exogenous compounds. Assays were supported by video microscopy observations of spore behaviour. The bioassays were used to ascertain the importance of cues (chemical, physico-chemical, biological (microbial biofilms) and topographical) in settlement and adhesion of *Enteromorpha* spores. Microtopographies were fabricated in PDMS by collaborators.

ACCOMPLISHMENTS: Standardised protocols were developed for a reliable quantitative bioassay for zoospore settlement and adhesion (Callow et al., 1997). The assays formed the basis of subsequent extensive studies (13 primary publications) during which many novel features of the settlement and adhesion processes were demonstrated. This includes their use in parallel molecular programmes funded by other agencies including the production of anti-adhesive antibody probes to characterise the spore adhesive (Callow et al., 2000a, Callow et al., 2001, Stanley et al., 1999).

Scatchard-type analyses of adhesion data revealed interesting spore density-dependent affects with positive co-operativity being observed at low densities and negative co-operativity at high densities. The significance of these results is that they suggest the possibility of inter-spore or spore-surface communication (Callow et al., 1997).

The most critical event in surface colonisation is the selection of a suitable surface on which to settle by a motile spore since commitment to adhesion results in permanent attachment. High resolution video microscopy identified a number of novel features of the spore attachment process (Callow et al., 1997). Surface sensing appears to proceed via the apical papilla, not the flagella. The spore spins on the apical papilla during a phase of 'surface sensing' and temporary adhesion. If the surface is 'inhospitable', it swims away to seek a more favourable attachment site. Permanent adhesion is moderated by the discharge of adhesive from vesicles present in the swimming spore. During and immediately after adhesive discharge, the spore undergoes 'space-

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filling' amoeboid-like movements in order to maximise adhesion to the substratum. Gregarious settlement (settlement in groups) is frequently observed. Subsequent detachment studies showed that spore groups are more strongly attached than single spores, thereby enhancing survival in a turbulent environment (Finlay et al., 2002).

To investigate the influence of surface physico-chemical properties on spore settlement we have made extensive use of model surfaces in the form of alkanethiol self-assembled monolayers (SAMs) (collaboration with Prof G Lopez, UNM) in preference to diverse substrates commonly used. SAMs of different wettability but uniform charge (uncharged) revealed a positive correlation between the number of spores that attached and increasing hydrophobicity (Callow et al., 2000b). However, a negative correlation was found between the strength of spore attachment and increasing wettability (Finlay et al., 2002). Spores responded differently to (COOH-)SAMs compared to (OH-SAMs) of equivalent wettability suggesting the involvement of parameters such as charge in spore attachment (Ista et al., in preparation).

We have shown that *Enteromorpha* spores respond to a number of external chemical cues. They are strongly attracted to trace quantities of certain solvents and to dibutyltin dilaurate, the curing agent used in commercial foul-release silicones. The cue appears to be the fatty acid (lauric acid) component of the molecule and we subsequently showed that spore attraction increased with the number of carbon atoms up to a maximum at C₁₄/C₁₅ (Callow & Callow, 1998b). This study laid the foundation for subsequent detailed explorations of natural chemical cues produced by *Enteromorpha* and by biofilm organisms (see below).

In the search for natural, settlement-modifying chemical cues produced by *Enteromorpha* lipophilic fractions of vegetative plants and swimming spores were investigated by LC-MS and bioassay methods. We showed that methanol and acetone extracts promoted and inhibited spore settlement respectively. Separation of both extracts by HPLC revealed many peaks some of which either inhibited or stimulated spore settlement. A number of closely related peaks with inhibitory activity were analysed by MS and NMR. Molecular masses ranged from 737.5 - 769.5 which is consistent with structures based on MGDG (monogalactosyldiacylglycerol) with differing acyl chains. In parallel studies on high molecular weight, water-soluble cues, 2 protein fractions with inhibitory and stimulatory effects on spore settlement were identified by SDS-PAGE and FPLC. The 180 kDa (native) inhibitor protein runs at 58 kDa on denaturing gels. Tryptic fragments were analysed by MALDI-QTOF but peptide sequence data were equivocal, hence the protein awaits further characterisation.

The response of spores to topographic cues was investigated via collaboration with Prof. A B Brennan (Florida). Microtextures fabricated in polydimethyl siloxane (PDMS) showed that spores settle preferentially in depressions (pits & grooves) and against features protruding from the surface (Callow et al., 2002, Wilkerson et al., 2001). Settlement of spores was much greater on patterns that were based on valley/ridge designs compared to pillars and that were 5 µm deep/high compared to ones 1.5 µm deep/high. Most spores attached in the angle between the valley floor and wall, an area of minimum energy. The addition of oils reduced settlement in the 5 µm wide valleys but the effect of oils were less marked on the patterns with lower profile (1.5 µm) features and no combinations of features and oil reduced spore settlement below that on the equivalent smooth, flat surface.

In studies on the role of cues presented by biofilm bacteria, we showed that natural mixed species biofilms promoted spore settlement and spores were preferentially associated with microcolonies (Joint et al., 2000). To examine the specificity of this response individual strains of bacteria were isolated from rocks and *Enteromorpha* plants and identified

by 16S rDNA sequencing (Patel et al., 2003). Biofilms of all strains were examined for their effect on spore settlement. Results showed that the effect of bacterial strains on spore settlement was strain- but not taxon-specific and activity varied with the age and density of the biofilm. The results suggest the possibility of prokaryotic-eukaryotic signalling and in associated work we have subsequently shown that one type of cue is the acylated homoserine autoinducer compounds that many bacteria produce in the 'quorum-sensing' process involved in biofilm formation (Joint et al., 2002). This is the first reported example of molecular cross-talk between prokaryotes and eukaryotes based on this particular type of molecule.

CONCLUSIONS: These studies provide the most comprehensive description and quantitative analysis of the settlement and adhesion processes and the roles of surface-associated cues, of any soft-fouling species to date. We have shown that *Enteromorpha* spores respond to a variety of cues including chemical, physico-chemical, biological (microbial biofilm) and topographic. Major new insights have been gained on communication processes that may be involved in the early stages of developing a micro-fouling community. Compounds that inhibit spore settlement could be employed in coatings to deter fouling. Understanding which factors promote settlement will inform the development of novel control strategies. Of immediate practical importance, our work has shown that in order to minimise fouling by *Enteromorpha*, surfaces should be smooth, since surface defects of a few microns amplitude will attract and shelter swimming algal spores. Coatings that contain known spore settlement promoters should also be avoided. The assays developed during this programme form the basis of test methods being used in a parallel ONR-funded programme to screen coatings for inhibition of algal settlement and foul-release potential.

SIGNIFICANCE: *Enteromorpha* is the most important macroalga that fouls ships and submarines whether coated with conventional copper-based paints or foul-release silicones. Since the occurrence of ship hulls fouled by *Enteromorpha* is currently increasing as a consequence of the increased deployment of TBT-free coatings, it is important to understand the processes that control and moderate spore settlement and adhesion. The most critical event in surface colonisation is the selection of a suitable surface by a motile spore since commitment to adhesion results in permanent attachment. Understanding these processes is fundamental to the development of novel coatings designed to prevent or minimise attachment.

PATENT INFORMATION: None

AWARD INFORMATION: MEC was promoted to Senior Research Fellow

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